Alt red Cytokine Export and Apoptosis in Mice Deficient in Interleukin-1β Converting Enzyme

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The interleukin-1β (IL-1β) converting enzyme (ICE) processes the inactive IL-1β precursor to the proinflammatory cytokine. Adherent monocytes from mice harboring a disrupted ICE gene ($ICE^{-/-}$) did not export IL-1β or interleukin-1α (IL-1α) after stimulation with lipopolysaccharide. Export of tumor necrosis factor–α and interleukin-6 (IL-6) from these cells was also diminished. Thymocytes from $ICE^{-/-}$ mice were sensitive to apoptosis induced by dexamethasone or ionizing radiation, but were resistant to apoptosis induced by Fas antibody. Despite this defect in apoptosis, $ICE^{-/-}$ mice proceed normally through development.

The cytokine IL-1β plays a pivotal role in acute and chronic inflammation, bone resorption, myelogenous leukemia, and other pathological processes (*1*). IL-1β is synthesized as a 31-kD precursor devoid of a conventional signal sequence (*2*) and is processed to its proinflammatory 17-kD form by ICE, a cysteine protease with substrate cleavage specificity for Asp-X (*3*). ICE itself is synthesized principally in monocytes as an inactive proenzyme that autoprocesses to an active tetramer composed of two 10-kD and two 20-kD subunits (*4, 5*). With the cloning of the *Caenorhabditis elegans* cell death gene *ced-3* (*6*), ICE was recognized to be a member of a new subfamily of cysteine proteases. ICE and CED-3 show only 28% sequence conservation overall, but their active site residues are completely conserved (*5, 6*).

Although the physiological functions of the mammalian ICE homologs are unknown, overexpression of ICE and ICE homologs in transfected cell lines induces apoptosis (*7, 8*). This effect is reduced when ICE is coexpressed with Bcl-2, a mammalian oncogenic protein that is a general suppressor of apoptosis (*9*). Further, transfection of chicken dorsal ganglion cells with CrmA, a serpin-like inhibitor of ICE (*10*) and potentially of ICE homologs, protects these cells from apoptosis induced by depletion of nerve growth factor (*11*).

To probe the physiological functions of ICE, we disrupted the murine ICE gene in D3 embryonic stem (ES) cells by replacing part of exons 6 and 7 (Fig. 1A) with a neomycin resistance gene cassette (*12, 13*). Chimeric mice were obtained by injection of mutant ES cells into C57BL/6 blastocysts, and the chimeric males were mated with C57BL/6 mice. Interbreeding of the heterozygous mice generated the expected mendelian 1:2:1 ratio of wild-type ($ICE^{+/+}$), heterozygous ($ICE^{+/-}$), and homozygous ($ICE^{-/-}$) mutant mice. Homozygous mice with two copies of the disrupted ICE gene

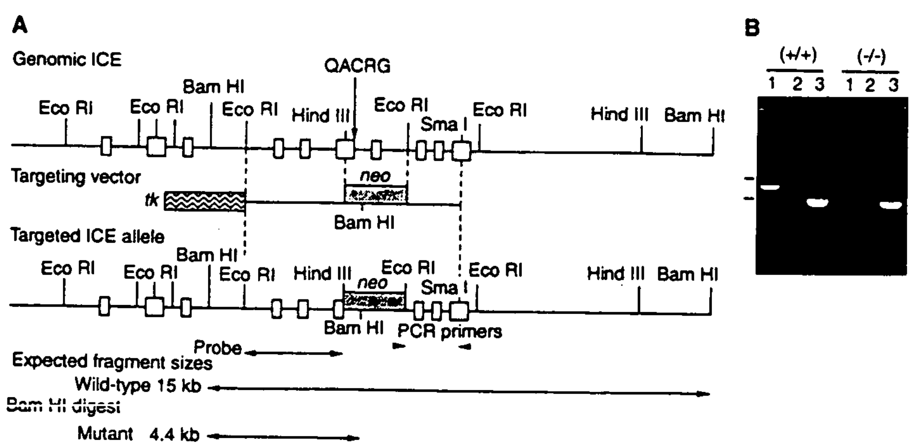

Fig. 1. Disruption of the murine ICE gene by homologous recombination (*13*). (**A**) Restriction maps of the murine ICE locus, the targeting vector, and the mutant ICE locus. (**B**) RT-PCR analysis of mRNA from $ICE^{+/+}$ and $ICE^{-/-}$ mice with oligonucleotide primers (*13*) specific for wild-type ICE cDNA (1), mutated ICE cDNA (2), and β-actin cDNA (3). The lines on the left indicate the positions of 984-bp (upper) and 738-bp (lower) markers.

were identified by Southern blots of genomic DNA, and the absence of ICE mRNA in $ICE^{-/-}$ mice was confirmed by reverse transcription–polymerase chain reaction (RT-PCR) analysis (Fig. 1B). The $ICE^{-/-}$ mice were healthy and fertile, and had no gross abnormalities in appearance, body weight, or organ size for at least the first 16 weeks of life. The apparently normal phenotype of $ICE^{-/-}$ mice suggests that ICE expression is dispensable for development.

Several serine proteases can process the IL-1β precursor to bioactive forms (*14*), but ICE is the only mammalian protease identified that generates the mature 17-kD cytokine with the naturally occurring Ala^{118} amino-terminus (*3*). A tetrapeptide inhibitor of ICE [Ac-Tyr-Val-Ala-Asp-CHO; inhibition constant (K_i) = 0.7 nM] blocks IL-1β processing and secretion from stimulated human monocytes or murine leukocytes [IC_{50} (the amount required to inhibit activity by 50%) ~ 1.5 μM] (*4*, *15*). This inhibitor, however, may not be completely selective for ICE and may also inhibit the proteolytic activities of ICE homologs.

To investigate the role of ICE in cytokine release, we examined $ICE^{+/+}$ and $ICE^{-/-}$ monocytes induced by lipopolysaccharide (LPS) and LPS plus nigericin. LPS is a bacterial endotoxin that induces monocytes to produce several cytokines including IL-1β, IL-1α, tumor necrosis factor–α (TNF-α), and IL-6. Nigericin is a K^+-H^+ ionophore that alters K^+ homeostasis and activates a plasma membrane adenosine triphosphatase (ATPase). Nigericin treatment after LPS stimulation enhances processing of the IL-1β precursor and export of mature 17-kD IL-1β from murine and human monocytes (*16*). In $ICE^{+/+}$ monocytes, LPS stimulated IL-1β export into the medium (39 ± 28 pg/ml) and LPS plus nigericin treatment significantly enhanced IL-1β export (140 ± 72 pg/ml, $P < 0.02$) (Table 1). In contrast, $ICE^{-/-}$ monocytes did not release any detectable IL-1β after stimulation with LPS or LPS plus nigericin, and no processed IL-1β was detectable in cell lysates from these cultures (Table 1). This observation establishes the critical role of ICE in processing and export of mature IL-1β.

Treatment with LPS and LPS plus nigericin also enhanced IL-1α release by $ICE^{+/+}$ monocytes (318 ± 186 pg/ml, $P < 0.02$) (Table 1). IL-1α binds to the same cellular receptors as IL-1β (*1*) and is also synthesized as a precursor, but pre–IL-1α is not a substrate for ICE (*3*). Surprisingly, $ICE^{-/-}$ monocytes did not release IL-1α after treatment with LPS plus nigericin, although the intracellular concentration of this cytokine was substantial (115 ± 38 pg/ml). These results implicate ICE as a mediator of IL-1α release from monocytes. Export of IL-1β and IL-1α may involve a common molecular assembly (*17*). For example, ICE may associate with the nigericin-stimulated K^+,H^+-ATPase for transport of IL-1α and

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Table 1. Cytokine secretion by adherent monocytes from $ICE^{+/+}$ and $ICE^{-/-}$ mice. Adherent monocytes were isolated from $ICE^{+/+}$ and $ICE^{-/-}$ mice and treated as in (*29*). Cytokines were quantitated in supernatants or cell lysates by an ELISA specific for murine IL-1β, IL-1α, TNF-α, and IL-6. The IL-1α ELISA recognizes both precursor and processed forms. The IL-1β ELISA is highly specific for mature IL-1β and shows <0.2% cross-reactivity against the murine IL-1β precursor.

Cytokine	Stimulus	Supernatant (S) or cell lysate (L)	Cytokine concentration (pg/ml)	
			$ICE^{+/+}$	$ICE^{-/-}$
IL-1β	None	S	0	0
	LPS	S	39 ± 28	0
		L	46 ± 46	0
	LPS + nigericin	S	140 ± 72	0
		L	52 ± 35	0
IL-1α	None	S	0	0
	LPS	S	117 ± 76	10 ± 13
		L	272 ± 175	100 ± 47
	LPS + nigericin	S	318 ± 186	0
		L	201 ± 163	115 ± 38
TNF-α	None	S	0	0
	LPS	S	531 ± 94	318 ± 10
IL-6	None	S	0	0
	LPS	S	2387 ± 190	1032 ± 803

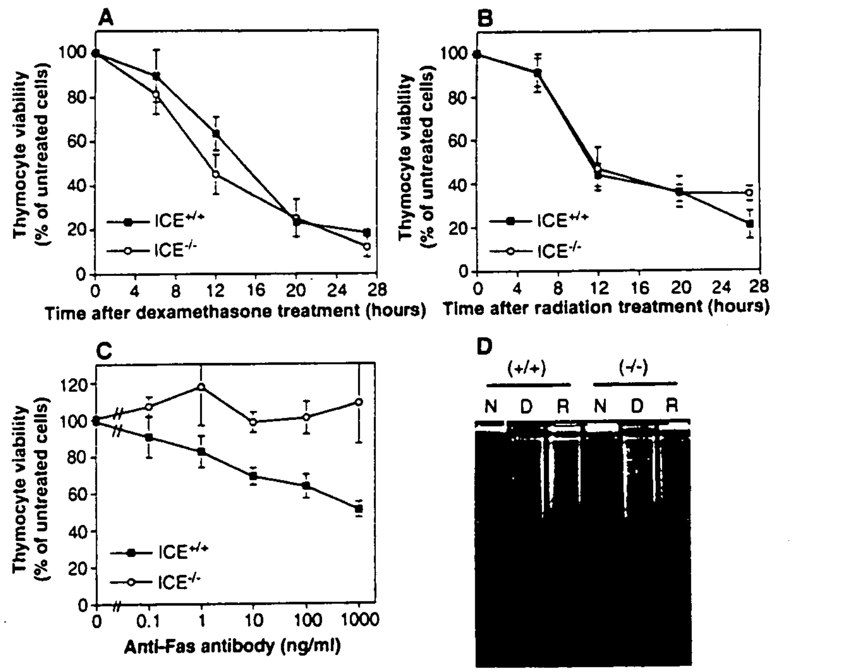

Fig. 2. Apoptosis in thymocytes from $ICE^{+/+}$ and $ICE^{-/-}$ mice. Thymocytes were treated with 1 μM dexamethasone (Sigma) (**A**); 500 cGy of ionizing radiation (**B**); or a hamster antibody to murine Fas (Pharmingen, San Diego) (**C**). Thymocytes were isolated from 5- to 6-week old mice and cultured at a cell density of 10^6 cells per milliliter in 24-well plates. Cells were incubated at 37°C and sampled for viability measurement at the time points indicated. For anti-Fas treatment, cells were incubated for 24 hours with antibody at the concentrations indicated. Cell viability was determined by trypan blue exclusion. Values represent the average viability from three independent wells (± SD) and are normalized to the percentage of viable cells remaining in the untreated cultures. Two independent experiments showed similar results. (**D**) Agarose gel electrophoresis of total DNA from thymocytes that received no treatment (N), or that were treated with 1 μM dexamethasone (D) or 500 cGy ionizing radiation (R). Genomic DNA was isolated from 10^6 thymocytes after 10 hours and subjected to electrophoresis.

IL-1β through the plasma membrane. Alternatively, ICE may interact with or activate other proteins, such as calpain (*18*), that may be involved in IL-1α processing and secretion. In contrast to IL-1α, TNF-α and IL-6 are secreted by ICE$^{-/-}$ monocytes, albeit at reduced levels (Table 1).

Overexpression of ICE or its homologs can induce apoptosis in cultured cells (*7, 8*). To determine if normal intracellular concentrations of ICE mediate apoptosis, we investigated the response of ICE$^{-/-}$ thymocytes to three apoptotic stimuli: glucocorticoid (*19*), ionizing radiation, and anti-Fas antibody (*20*). The Fas antigen is a cell surface protein in the TNF-α receptor superfamily that mediates apoptosis in activated T cells (*21, 22*). Fas is encoded by the gene responsible for a lymphoproliferative disorder (*lpr*) in mice. Mice that carry mutations in Fas develop lymphadenopathy and suffer from a systemic autoimmune disease (*23*). Thymocytes isolated from ICE$^{-/-}$ and ICE$^{+/+}$ mice were sensitive to dexamethasone- or radiation-induced apoptosis, as evaluated by cell viability and DNA fragmentation (Fig. 2). A monoclonal antibody to Fas triggered apoptosis in ICE$^{+/+}$ thymocytes in a dose-dependent manner, but did not induce apoptosis in ICE$^{-/-}$ thymocytes (Fig. 2). Analysis of ICE$^{+/+}$ and ICE$^{-/-}$ thymocytes by fluorescence-activated cell sorting (FACS) revealed no differences in expression of cell surface Fas antigen (Fig. 3). We also observed that a potent ICE inhibitor, Cbz-Val-Ala-Asp-(OEthyl)-[(2,6-dichlorobenzoyl)oxy]methyl-ketone, prevented Fas-triggered apoptosis of ICE$^{+/+}$ thymocytes in a dose-dependent manner (*24*).

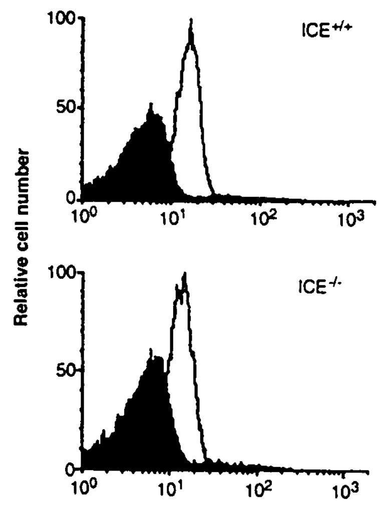

Fig. 3. Expression of Fas on ICE$^{+/+}$ and ICE$^{-/-}$ thymocytes. Cells (10^6) were stained first with 1 μg of the hamster antibody against murine Fas and then with a fluorescein isothiocyanate–conjugated goat antibody to hamster immunoglobulin G (E-Y Laboratories, San Mateo, California). Fas expression on the cell surface was analyzed by FACS. Shaded areas represent staining by the second antibody.

These results establish a role for ICE in Fas-mediated apoptosis of normal thymocytes. Glucocorticoid- and radiation-induced apoptosis of thymocytes presumably occur through different pathways that may involve intracellular serine proteases (*25*). The existence of multiple apoptotic pathways in thymocytes has been inferred previously from studies of mice deficient in Bcl-2. Mature T cells from *bcl-2*$^{-/-}$ mice showed increased sensitivity to apoptosis induced by glucocorticoids and ionizing radiation, but reduced sensitivity to apoptosis stimulated by an antibody to CD3 (*26*). Targeted expression of Bcl-2 in murine cortical (CD4$^-$CD8$^-$) thymocytes inhibited apoptosis induced by glucocorticoid, ionizing radiation, and anti-CD3, but did not inhibit negative selection of T cells (*27*). Similarly, the tumor suppressor protein p53 mediates apoptosis induced by ionizing radiation and etoposide, but not apoptosis induced by glucocorticoid and other stimuli (*28*).

Unlike the *lpr/lpr* mutation at the murine Fas locus (*23*), disruption of the ICE gene does not lead to autoimmune pathologies in mice within the first 16 weeks of life. This suggests that ICE$^{-/-}$ mice undergo normal clonal deletion of T cells recognizing endogenous superantigens. Given that interaction of Fas and its ligand is involved in programmed cell death after T cell activation (*22*), clonal deletion might be dependent on ICE homologs and their function in apoptotic pathways.

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13. A 2.5-kb Eco RI–Hind III fragment containing ICE exons 4 to 6 and a 1.3-kb Eco RI–Sma I fragment containing exons 8 to 10 (*30*) were subcloned with the neomycin resistance (*neo*) gene cassette (*25*) into a thymidine kinase (*tk*) gene-expressing plasmid to generate the targeting vector. The vector was linearized and introduced into D3 ES cells by electroporation. Sixty-three clones resistant to G418 (300 μg/ml) and gancyclovir (2 μM) were screened by PCR using an exon 10 primer (5′-GTACATAAGAATGAACTGGA-3′) and a *neo* cassette-specific primer (5′-TGCTAAAGCGCATGCTCCAGACTG-3′). One correctly targeted clone was confirmed by Southern blot analysis. Chimeric mice were generated from the mutant ES cell, and heterozygous mice (ICE$^{+/-}$) were interbred to obtain homozygous ICE$^{-/-}$ mice. Expression of ICE mRNA in ICE$^{+/+}$ and ICE$^{-/-}$ mice was analyzed by RT-PCR analysis using the following pairs of PCR primers: (i) ICE complementary DNA (cDNA)–specific primers to exon 3 (5′-GATTCTAAAGGAGGACATCC-3′) and exon 10 (5′-GTACATAAGAATGAACTGGA-3′) to generate an expected PCR product of 930 base pairs (bp); (ii) mutated ICE cDNA-specific primers of exon 3 and a 3′ region of the *neo* cassette (5′-GGGCCAGCTCATTCCTCCACT-3′) to generate an expected PCR product of 700 bp; and (iii) β-actin–specific primers (5′-CACCCTGTGCTGCTCACCGAGGCC-3′ and 5′-CCACACAGATGACTTGCGCTCAGG-3′) to generate an expected product of 700 bp. RT-PCR products were identified with DNA ladder markers of 123 bp (Gibco, Gaithersburg, MD).
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24. The ICE$^{+/+}$ thymocytes were incubated with 1 μg/ml anti-Fas antibody in the presence of 0.1, 1.0, or 10.0 μM of Cbz-Val-Ala-Asp-(OEthyl)-[(2,6-dichlorobenzoyl)oxy]methyl-ketone. Thymocyte viability was assessed 24 hours after treatment as in Fig. 2. Inhibition of apoptosis (IC_{50} ~ 1 μM) was determined in two independent experiments.
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29. Cell suspensions were prepared from spleens excised from ICE$^{+/+}$ or ICE$^{-/-}$ mice. Adherent monocytes were stimulated for 16 to 18 hours with 1 μg/ml phenol-extracted LPS (*Escherichia coli* strain 0111:B4; Sigma), and supernatants were harvested for quantitation of IL-1β. Cells from some cultures were washed once, incubated with 10 μM nigericin for an additional 30 min, and the supernatants harvested again. Viability of monocytes after nigericin stimulation was >98% as measured by trypan blue staining or lactate dehydrogenase activity in culture supernatants, indicating that minimal cytolysis had occurred during the experiment. IL-1β was quantitated in supernatants and cell lysates with an enzyme-linked immunosorbent assay (ELISA) specific for mature murine IL-1β (PerSeptive Diagnostics,

Cambridge, MA). We determined the specificity by testing the reactivity of purified recombinant murine IL-1β precursor in the ELISA. At concentrations of 20 pg/ml and 10 ng/ml, respectively, mature IL-1β and precursor IL-1β were recognized equally, indicating a cross-reactivity of 0.2%. The IL-1α antibody was from Genzyme (Cambridge, MA), and the TNF-α and IL-6 antibodies were from Biosource International (Camarillo, CA).

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